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DESIGN AND SYNTHESIS OF THROMBIN SUBSTRATES WITH MODIFIED KINETIC PARAMETERS

Dirk T.S. Rijkers[1,2], Simone J.H. Wielders[1], Godefridus I. Tesser[2] and H.Coenraad Hemker[1]
[1]University of Limburg, Faculty of Medicine, Department of Biochemistry, P.O. Box 616, 6200 MD Maastricht, The Netherlands
[2]Catholic University of Nijmegen, Department of Organic Chemistry, Toernooiveld, 6525 ED Nijmegen, The Netherlands

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Abstract: For the continuous registration of thrombin formation in plasma (1), selective thrombin substrates are required, that show moderate binding affinities (high K_m) and low turnover numbers (low k_{cat}). Previously we have used SQ68 (CH_3O-CO-CH_2-CO-Aib-Arg-pNA) for this purpose. In order to find more substrates suitable for this application, we synthesized a series of 25 peptide *p*-nitroanilides. As lead structures SQ68 and S2238 (H-D-Phe-Pip-Arg-pNA) were used. By introduction of specific structure modifications we tried to alter the kinetic data in the required direction. The modifications were designed on basis of existing knowledge on the structure of the thrombin active-site and its surroundings. We indeed obtained a number of substrates with the kinetic constants in the desired range.

For the continuous registration of thrombin generation and the determination of the endogenous thrombin potential (ETP: area under the thrombin generation curve) it is necessary to dispose of specific thrombin substrates that are converted at a slow rate so as not to be exhausted when added to clotting plasma (1). The suitable substrate that is now available (Serbio SQ68), was found by accident. Published modeling and X-ray analyses of the thrombin molecule offer insight into the three-dimensional form of its active-site and suggest modifications of existing thrombin substrates that may serve to tune the kinetic parameters in the required direction. In this article we report the results of a synthetic search for specific, slow reacting thrombin substrates.
Current research focusses on H-D-Phe-Pip-Arg-pNA (S2238) as a lead structure in the development of highly specific thrombin inhibitors. Derivatives of this structure containing aldehyde- (2), chloromethylketone- (3), aminoboronic acid- (4), ketomethylene- (5) and aminophosphonic acid- (6) functions were described as potent and specific transition-state

Key words: chemical synthesis, chromogenic substrates, thrombin substrates.
Abbreviations: Abbreviations used for amino acids and for the designations of peptides follow the rules of the IUPAC-IUB Joint Commission on Biochemical Nomenclature in Eur. J. Biochem. *138*, 9-37, 1984.
Corresponding author: Dr. H.C. Hemker, University of Limburg, Faculty of Medicine, Department of Biochemistry, P.O. Box 616, 6200 MD Maastricht, The Netherlands, Phone: +3143881675, Fax: +3143670988.

analogue inhibitors of thrombin. All these inhibitors contain an arginine in the P1 position corresponding to the primary specificity of thrombin.
Recent publications showed that inhibitors derived from H-D-Phe-Pip-Arg that, at position P1, contain an amino acid with a neutral side chain show enhanced selectivity towards thrombin. Also, substances in which the positively charged side chain of arginine is replaced by a neutral side chain, containing aldehyde- (7), aminoboronic acid- (8) and aminophosphonic acid- (9) functions bind tightly to thrombin. This result is somewhat surprising, because it was believed that electrostatic interaction between the positively charged guanidino group of inhibitors and substrates, and the negatively charged Asp189 residue in the primary specificity pocket of thrombin is essential for complex formation. The crystal structure of human α-thrombin complexed with H-D-Phe-Pro-Arg-CH_2Cl, however, showed that the active-site consists of two important binding regions: the hydrophobic binding pocket, S2 subsite, which interacts preferentially with proline residues, and the aryl-binding pocket (S3) in which aromatic stacking interactions (D-phenylalanine) are of major importance for strong binding affinity (10-11). The fact that inhibitors containing the H-D-Phe-Pro sequence with a modified P1 residue still bind to thrombin can be explained in terms of these binding interactions. Also, the inhibitors containing amino acids with neutral side chains at the P1 position show enhanced selectivity towards thrombin compared to other serine proteases with an arginine as primary determinant of specificity. Comparison of the S1 binding pockets of thrombin, trypsin and plasmin showed that the S1 binding pocket of thrombin is the most hydrophobic one. This explains why a neutral side chain at P1 would be better accommodated by thrombin than by trypsin or plasmin. The improvement of selectivity is paid by a decrease of potency as a result of a loss in electrostatic binding interactions with Asp189.
These findings motivated us to try and synthesize pNA-substrates with maintenance of the H-D-Phe-Pip(Pro) sequence and in which the P1 amino acid residue is varied, see table I.

Another starting point was CH_3O-CO-CH_2-CO-Aib-Arg-pNA (SQ68) (12). At position P2 and P3, this substrate has not the optimal structural elements for interaction with thrombin. We started an investigation into a structure-activity relationship to obtain substrates selective for thrombin, in which a dipeptide is the smallest determinant of selectivity, the case being modified at its N-terminus to mimic a P3 residue. From modeling studies (13-14) and homology model-building (15) it has been concluded that the β-naphtalenesulfonyl function interacts with thrombin in a highly specific manner, whereas interaction with other serine proteases is minimal. The *p*-toluenesulfonyl group showed the same binding interactions as the β-naphthalenesulfonyl moiety, albeit to a somewhat lesser extent (16). These findings were confirmed by X-ray studies of thrombin-complexes formed with active-site directed inhibitors containing these structural elements (17-18). We used both functions to arrive at more selective thrombin substrates.

EXPERIMENTAL PROCEDURES

General
^1H Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM 100 and Bruker AM 400 spectrometers (Palo Alto, USA). As an internal standard the residual solvent peak was used. Chemical shifts are given in part per million (ppm). Optical rotations were measured on a Perkin Elmer 241 polarimeter in a 10 cm cuvette at room temperature. Melting points were determined with a Büchi melting point apparatus (Tottoli). Thin layer chromatography (TLC) was performed on Merck Silicagel $60F_{254}$ plates (Darmstadt, Germany), and column chromatography on Merck Kieselgel 60, 70-230 Mesh ASTM. Spots were detected by UV-fluorescence quenching, ninhydrine (free amino functions), chlorine/TDM (4,4'-tetramethyl-diaminodiphenylmethane, NH groups), Barton's- (hydrazides) and Sakaguchi's (arginine residues) reagent. Methanol was refluxed on magnesium turnings for two hours, distilled and stored on 3Å molsieves. Pyridine was distilled over KOH pellets and stored on 4Å molsieves. *tert.*-Butyloxycarbonyl (Boc)-amino acids were synthesized by the method of Schnabel (19). Amino acid *p*-nitroanilides were prepared as described by us (20).

TABLE I

Substrates of the Type H-D-Phe-Y-X-pNA and Y-X-Arg-pNA with S2238 respectively SQ68 as Lead Structures.

H-D-Phe-Y-X-pNA				
Y	X	Code	Derived from	Code
			H-D-Phe-Pip-Arg-pNA	1
Pip	Lys	**1a**		
Pip	Orn	**1b**		
Pip	Glu	**1c**		
Pip	Nle	**1d**		
			H-D-Phe-Pro-Arg-pNA	2
Pro	Lys	**2a**		
Pro	Nle	**2b**		

Y-X-Arg-pNA				
Y	X	Code	Derived from	Code
			H-Gly-Arg-pNA	3
MZ	Gly	**3a**		
MMZ	Gly	**3b**		
DMMZ	Gly	**3c**		
DEMZ	Gly	**3d**		
Nas	Gly	**3e**		
Tos	Gly	**3f**		
			H-Ala-Arg-pNA	4
MZ	Ala	**4a**		
MMZ	Ala	**4b**		
DMMZ	Ala	**4c**		
			H-D-Ala-Arg-pNA	5
MZ	D-Ala	**5a**		
			H-Val-Arg-pNA	6
MZ	Val	**6a**		
			H-D-Val-Arg-pNA	7
MZ	D-Val	**7a**		

Unprotected amino functions are suitably protonated (HCl) to give neutral solutions in plain water.

Malonic acid and dimethylmalonic acid were converted to their corresponding dimethyl esters by heating the dicarboxylic acid with an excess of methanol in the presence of concentrated sulfuric acid. Dimethyl methylmalonate was prepared by generating the mono-sodio derivative of dimethylmalonate which reacts with methyl iodide *via* a S_N2 mechanism. The corresponding half esters were prepared by controlled partial hydrolysis of the diester with one equivalent of potassium hydroxide. The monomethyl ester of diethylmalonic acid was obtained in a direct way by refluxing the acid in an excess of methanol with sulfuric acid as catalyst. Dimethylmalonic acid and L-pipecolic acid were from Janssen Chimica (Geel, Belgium), diethylmalonic acid was from Aldrich (Bornem, Belgium). TDM, naphthalene-2-sulfonyl chloride, toluene-4-sulfonyl chloride and D-alanine were obtained from Fluka (Buchs, Switzerland). Boc-Nle-OH, and Fmoc-Orn(Boc)-OH were from Bachem (Bubendorf, Switzerland). D-Phenylalanine was a generous gift of Dr. J. Kamphuis (DSM Research, Geleen, The Netherlands).

Syntheses

Boc-D-Phe-Pip-N_2H_3 :

To a solution of Boc-D-Phe-OH (19.88 g, 75 mmol) in EtOAc (350 mL) was added HCl.H-Pip-OMe (21) (13.46 g, 75 mmol), HOBt (12.62 g, 82.5 mmol) and NMM (8.75 mL, 78.7 mmol). The obtained suspension was cooled on ice and DCC (16.22 g, 78.7 mmol) was added. After stirring for two hrs at 0°C and 16 hrs at room temperature, DCU was filtered off. The EtOAc solution was washed with 2N $KHSO_4$, H_2O, saturated $NaHCO_3$ and brine (four times 50 mL each). The EtOAc layer was dried (Na_2SO_4), filtered and evaporated *in vacuo*, yielding a clear oil, yield: 21.52 g (74%). This product was checked by TLC on purity and directly used for further synthesis (R_f(CH_2Cl_2/MeOH 9:1 v/v): 0.87). The protected dipeptide methyl ester was dissolved in MeOH (150 mL) and $N_2H_4.H_2O$ (13.5 mL, 278 mmol) was added. This reaction mixture was left for three days at room temperature. The formed precipitate was filtered and dried; yield: 15.01 g (70%), R_f(CH_2Cl_2/MeOH 9:1 v/v): 0.54, $[\alpha]_D$ = -77.1° c = 0.98 DMF, mp: 202-204°C, ^{1}H NMR (CD_3OD): δ = 1.15-1.38 (m, 6H, CH_2-Pip (3 x 2H)); 1.44 (s, 9H, Boc); 3.08/3.18 (dm, 2H, β-CH_2-Phe); 3.28 (m, 2H, N-CH_2-Pip); 4.58 (m, 1H, α-CH-Pip); 4.74 (m, 1H, α-CH-Phe); 7.20-7.33 (m, 5H, arom Phe).

Boc-D-Phe-Pip-X-pNA

a) X = Orn(Boc):

Boc-D-Phe-Pip-N_2H_3 (0.39 g, 1.0 mmol) was dissolved in DMF (10 mL) and cooled to -20°C. To this solution was added 2.3 M HCl/EtOAc (1.20 mL, 2.75 mmol) followed by *tert.*-BuONO (0.114 mL, 1.20 mmol). This reaction mixture was stirred for 15 min at -20°C. (After this period the azide-formation was complete.) The acid solution was neutralized by adding DIPEA (0.48 mL, 2.77 mmol). H-Orn(Boc)-pNA (0.328 g, 1.0 mmol) was added. This reaction mixture was kept on neutrality by adding DIPEA at regular time intervals. The obtained reaction medium was allowed to react during 16 hrs at 0°C. After this period the DMF phase was removed *in vacuo* and the oily residue was dissolved in EtOAc (10 mL). The EtOAc solution was washed with H_2O, 2N $KHSO_4$, H_2O saturated $NaHCO_3$ and brine (three times 5 mL each). The EtOAc solution was dried (Na_2SO_4), filtered and evaporated *in vacuo*. Boc-D-Phe-Pip-Orn(Boc)-pNA was purified by column chromatography on silica gel with CH_2Cl_2/MeOH 98:2 v/v as eluens. Yield: 0.525 g (74%), R_f(CH_2Cl_2/MeOH 98:2 v/v): 0.22, $[\alpha]_D$ = -64.8° c = 0.13 MeOH, ^{1}H NMR (CD_3OD): δ = 1.26-1.48 (bm, 6H, CH_2-Pip (3 x 2H)); 1.34 (s, 9H, Boc); 1.41 (s, 9H, Boc); 1.56/1.65 (dm, 2H, γ-CH_2-Orn); 1.78/1.99 (dm, 2H, β-CH_2-Orn); 2.97 (m, 2H, β-CH_2-Phe); 3.07 (m, 4H, δ-CH_2-Orn/N-CH_2-Pip); 4.48 (m, 2H, α-CH-Orn/α-CH-Pip); 4.79 (m, 1H, α-CH-Phe); 7.25-7.32 (m, 5H, arom Phe); 7.86/7.88-8.18/8.20 (dd, 4H, arom pNA).

b) X = Glu(O^{t}Bu)

Obtained as described for a).

Yield: 0.422 g (62%), R_f(CH_2Cl_2/MeOH 98:2 v/v): 0.44, $[\alpha]_D$ = -78.5° c = 0.33 MeOH, ^{1}H NMR (CD_3OD): δ = 1.26-1.54 (bm, 8H, β-CH_2-Glu/CH_2-Pip (3 x 2H)); 1.33 (s, 9H, O^{t}Bu); 1.43 (s, 9H, Boc); 2.38 (m, 2H, γ-CH_2-Glu); 2.96 (m, 2H, β-CH_2-Phe); 3.08 (m, 2H, N-CH_2-Pip); 4.53 (dd, 1H, α-CH-Glu); 4.59 (m, 1H, α-CH-Pip); 4.82 (m, 1H, α-CH-Phe); 7.26-7.33 (m, 5H, arom Phe); 7.89/7.91-8.20/8.22 (dd, 4H, arom pNA).

nHCl.H-D-Phe-Pip-X-pNA

The protected tripeptide was dissolved in AcOH (1mmol in 5 mL). To the solution 2.3 M HCl/EtOAc (10 mL for 1 mmol) was added and the reaction mixture was stirred until deprotection was complete. The acid was quenched with *tert.*-BuOH (10 mL) and evaporated *in vacuo*. The residue was coevaporated with *tert.*-BuOH (twice) and with MeOH (once). The residue was dissolved in H_2O and lyophilized. Crude tripeptides were purified by counter current distribution with BuOH/AcOH/H_2O 4:1:5 v/v/v as solvent system.

*a) n = 2, X = Orn (**1b**):*

Yield: 0.315 g (96%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.42 (K = 0.71), $[\alpha]_D$ = -96.7° c = 0.18 MeOH, ^{1}H NMR (CD_3OD): δ = 1.29-1.49 (m, 6H, CH_2-Pip (3 x 2H)); 1.62-1.77 (dm, 2H, γ-CH_2-Orn); 2.00-2.15 (dm, 2H, β-CH_2-Orn); 2.98 (m, 6H, β-CH_2-Phe/N-CH_2-Pip/δ-CH_2-Orn); 4.34 (m, 1H, α-CH-Pip); 4.46 (m, 1H, α-CH-Orn); 4.57 (m, 1H, α-CH-Phe); 7.23-7.35 (m, 5H, arom Phe); 7.85/7.87-8.20/8.22 (dd, 4H, arom pNA).

*b) n = 1, X = Glu (**1c**):*
Yield: 0.158 g (64%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.67 (K = 5.15), $[\alpha]_D$ = -107.4° c = 0.22 MeOH, ^{1}H NMR (CD_3OD): δ = 1.11-1.35 (m, 6H, CH_2-Pip (3 x 2H); 2.03 (m, 2H, β-CH_2-Glu); 2.26/2.42 (dm, 2H, γ-CH_2-Glu); 2.82/3.55 (dm, 2H, N-CH_2-Pip); 3.11/3.24 (dm, 2H, β-CH_2-Phe); 4.17 (m, 1H, α-CH-Glu); 4.52 (m, 1H, α-CH-Pip); 4.82 (m, 1H, α-CH-Phe); 7.26-7.41 (m, 5H, arom Phe); 7.84/7.86-8.19/8.22 (dd, 4H, arom pNA).

Boc-X-Arg-pNA.HCl
a) X = Gly:
Boc-Gly-OH (1.75 g, 10 mmol), 2HCl.H-Arg-pNA (3.67 g, 10 mmol), HOBt (1.60 g, 10.1 mmol) and NMM (1.10 mL, 10.0 mmol) were dissolved in DMF (75 mL). This reaction mixture was cooled on ice and DCC (2.16 g, 10.5 mmol, 1.05 eq.) was added. After stirring for 1 hr at 0°C and 16 hrs at room temperature, DCU was filtered off and DMF was removed under reduced pressure. The residue was purified by counter current distribution with BuOH/AcOH/H_2O 4:1:5 v/v/v (K = 1.84) as the solvent system. Pure product was lyophilized from AcOH. Yield: 4.29 g (88%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.64, $[\alpha]_D$ = -36.8° c = 0.63 MeOH.
b) X = Val:
Obtained as described for a).
Yield: 2.36 g (89%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.69 (K = 2.11), $[\alpha]_D$ = -44.4° c = 0.59 MeOH, ^{1}H NMR (CD_3OD): δ = 0.94-0.98 (dd, 6H, γ-CH_3/γ'-CH_3-Val); 1.44 (s, 9H, Boc); 1.70 (m, 1H, β-CH-Val); 1.82 (m, 2H, γ-CH_2-Arg); 2.03 (m, 2H, β-CH_2-Arg); 3.23 (m, 2H, δ-CH_2-Arg); 3.88 (d, 1H, α-CH-Val); 4.56 (m, 1H, α-CH-Arg); 7.84/7.86-8.20/8.22 (dd, 4H, arom pNA).

*2HCl.H-Val-Arg-pNA (**6**):*
As described for nHCl.H-D-Phe-Pip-X-pNA.
Yield: 0.401 g (86%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.40, $[\alpha]_D$ = -5.7° c = 0.21 MeOH, ^{1}H NMR (CD_3OD): δ = 1.05/1.07-1.08/1.10 (dd, 6H, γ-CH_3/γ'-CH_3-Val); 1.70-1.89 (m, 3H, γ-CH_2-Arg/β-CH-Val); 1.96/2.25 (dm, 2H, β-CH_2-Arg); 3.26 (m, 2H, δ-CH_2-Arg); 3.82 (d, 1H, α-CH-Val); 4.58 (m, 1H, α-CH-Arg); 7.85/7.87-8.20/8.22 (dd, 4H, arom pNA).

R-X-Arg-pNA.HCl
The monomethyl ester of the malonic acid derivative was coupled with DCC/HOBt in DMF with 2HCl.H-X-Arg-pNA in the presence of DIPEA for 1 hour at 0°C and 16 hrs at room temperature. After filtration, the clear DMF phase was evaporated *in vacuo*. The residue was purified by counter current distribution with the solvent system: BuOH/AcOH/H_2O 4:1:5 v/v/v. Pure product was lyophilized from H_2O.
*R = DEMZ, X = Gly (**3d**):*
Yield: 0.398g (74%), R_f(BuOH/AcOH/H_2O 4:1:1 v/v/v): 0.57 (K = 2.31), $[\alpha]_D$ = -29.8° c = 0.27 MeOH, ^{1}H NMR (D_2O): δ = 0.85 (t, 6H, CH_3-diethylmalonic acid (2 x 3H)); 1.59 (m, 4H, β-CH_2/γ-CH_2-Arg); 1.92 (q, 4H, ~CH_2~ diethylmalonic acid (2 x 2H)); 3.19 (m, 2H, δ-CH_2-Arg); 3.79 (s, 3H, OCH_3); 3.92 (s, 2H, CH_2-Gly); 4.24 (m, 1H, α-CH-Arg); 7.25/7.32-7.64/7.73 (dd, 4H, arom pNA).

Chromogenic substrates: The substrates were dissolved in distilled water to obtain 10 mM stock solutions. The concentration was determined at 316 nm using a molar extinction coefficient of 12,500 $Lmol^{-1}cm^{-1}$. Stock solutions were stored in the dark at 4°C.

Enzyme preparations: Pure human α-thrombin and human factor Xa samples were generous gifts of Dr. T. Lindhout (University of Limburg, Maastricht, The Netherlands).

Kinetic parameters: The hydrolysis experiments were run in buffer A (0.05 M Tris-HCl, 0.1 M NaCl, pH = 7.35 containing 0.5 g/L bovine serum albumin (Sigma, Bornem, Belgium)) at 37°C. The liberation of *p*-nitroaniline was monitored at 405 nm in a dual wavelength (405-546 nm) spectrophotometer made in our workshop, using a personal computer for data recording.

TABLE II

Kinetics of Hydrolysis of a Number of Defined Substrates by Human α-Thrombin and Human Factor Xa in Buffer A at 37 °C.

Entry	Substrate	h-α-thrombin			h-factor Xa		
		K_m µM	k_{cat} s^{-1}	k_{cat}/K_m $(Ms)^{-1}$	K_m µM	k_{cat} s^{-1}	k_{cat}/K_m $(Ms)^{-1}$
1	2HCl.H-D-Phe-Pip-Arg-pNA (**1**)#	6	237	3.95E7	174	8	4.60E4
2	2HCl.H-D-Phe-Pro-Arg-pNA (**2**)	3	28	9.33E6	690	19	2.75E2
3	2HCl.H-D-Phe-Pip-Lys-pNA (**1a**)	65	118	1.82E6	960	0.6	6.25E2
4	2HCl.H-D-Phe-Pro-Lys-pNA (**2a**)	35	90	2.57E6	-	-	-
5	2HCl.H-D-Phe-Pip-Orn-pNA (**1b**)	420	5	1.19E4	NH	-	-
6	HCl.H-D-Phe-Pip-Nle-pNA (**1d**)	943	11	1.17E4	NH	-	-
7	HCl.H-D-Phe-Pro-Nle-pNA (**2b**)	88	7	7.95E4	-	-	-
8	HCl.H-D-Phe-Pip-Glu-pNA (**1c**)	474	0.4	8.44E2	NH	-	-
9	2HCl.H-Gly-Arg-pNA (**3**)	2280	0.10	4.38E1	-	-	-
10	2HCl.H-Ala-Arg-pNA (**4**)	1140	0.07	6.14E1	-	-	-
11	2HCl.H-D-Ala-Arg-pNA (**5**)	4100	0.10	2.44E1	-	-	-
12	2HCl.H-Val-Arg-pNA (**6**)	1240	0.23	1.85E2	NH	-	-
13	2HCl.H-D-Val-Arg-pNA (**7**)	4250	0.3	7.06E1	-	-	-
14	MZ-Gly-Arg-pNA.HCl (**3a**)	2540	12	4.72E3	4700	55	1.17E4
15	MZ-Ala-Arg-pNA.HCl (**4a**)	1540	108	7.01E4	5600	52	9.29E3
16	MZ-D-Ala-Arg-pNA.HCl (**5a**)	1330	0.06	4.51E1	3100	0.09	2.90E1
17	MZ-Aib-Arg-pNA.HCl* (SQ68)	830	0.46	5.54E2	3940	2.9	7.38E2
18	MZ-Val-Arg-pNA.HCl (**6a**)	1000	14	1.40E4	2570	6	2.33E3
19	MZ-D-Val-Arg-pNA.HCl (**7a**)	1310	0.15	1.15E2	1820	0.13	7.14E1
20	MMZ-Gly-Arg-pNA.HCl (**3b**)	1740	7	4.02E3	6400	44	6.88E3
21	MMZ-Ala-Arg-pNA.HCl (**4b**)	670	17	2.54E4	7100	41	5.77E3
22	DMMZ-Gly-Arg-pNA.HCl (**3c**)	900	6	6.67E3	2840	14	4.93E3
23	DMMZ-Ala-Arg-pNA.HCl (**4c**)	570	4	7.02E3	-	-	4.93E3**
24	DEMZ-Gly-Arg-pNA.HCl (**3d**)	870	2	2.30E3	2320	5	2.16E3
25	Nas-Gly-Arg-pNA.HCl (**3e**)	NH	-	-	120	2	1.67E4
26	Tos-Gly-Arg-pNA.HCl (**3f**)	NH	-	-	230	9	3.91E4

NH: not hydrolyzed; *from reference 1; #substrate numbering as given in table I; **non Michaelis-Menten kinetics.

In a polystyrene microcuvette (total volume 500 µL), buffer A and substrate solution were added to obtain a final substrate concentration between 1 to 2000 µM. After 5 min of incubation at 37°C, enzyme solution was added to achieve a final concentration between 0.5 to 100 nM. The measurement was carried out in a thermostated cuvette-holder at 37°C. The Michaelis constant (K_m) and the catalytic constant (k_{cat}) were obtained by measuring initial reaction velocities at different substrate concentrations (see table II). The data obtained were fitted using a non-linear regression procedure to the Michaelis-Menten equation.

Continuous registration of the thrombin generation curve: The measurement is described in full detail in reference 1. Briefly, to 400 µL defibrinated plasma in a polystyrene microcuvette was added 110 µL buffer A, 20 µL phospholipid solution (phosphatidyl choline/phosphatidyl serine 8:2, final concentration 1.5 µM) and 30 µL substrate solution (final concentration 500 µM). After 4 min of incubation at 37°C, 20 µL of a recombinant tissue factor solution (final concentration 6 ng/mL) was added. Thrombin generation was started by adding 20 µL of a 0.5 M $CaCl_2$ solution (final concentration 16.7 mM); during the measurement the temperature was kept at 37°C. The

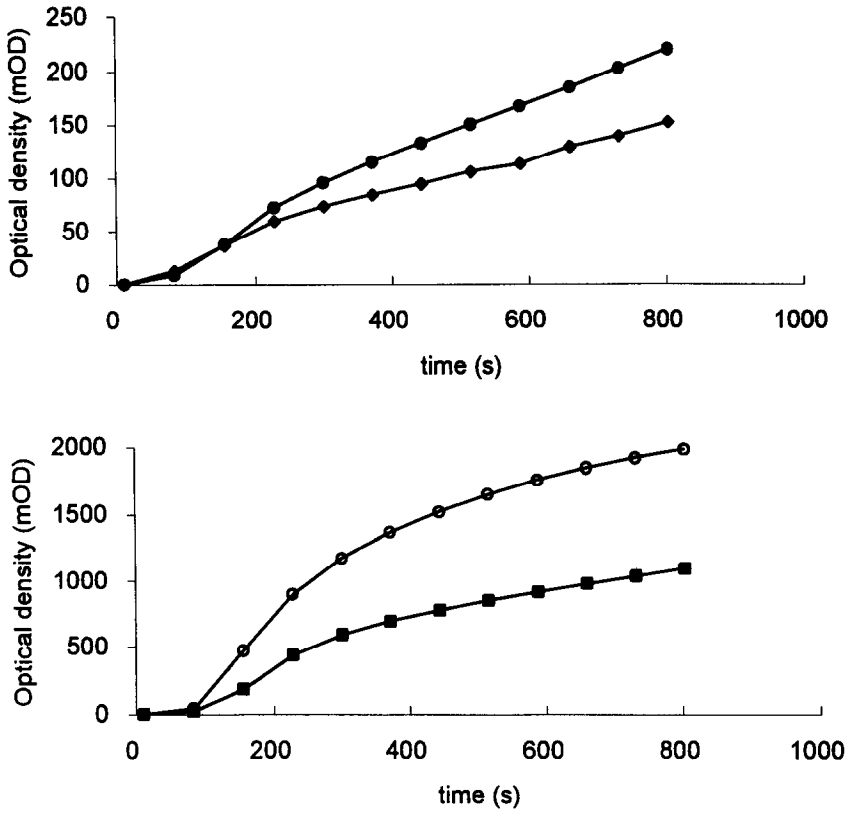

FIG. 1.

Continuous optical density curves measured with different substrates in tissue factor triggered plasma. Upper frame: ●: 2HCl.H-Val-Arg-pNA (**6**), ◆: MZ-Aib-Arg-pNA.HCl (SQ68, reference); lower frame: ○: 2HCl.H-D-Phe-Pip-Orn-pNA (**1b**), ■: DEMZ-Gly-Arg-pNA.HCl (**3d**). The optical density curve measured with HCl.H-D-Phe-Pip-Glu-pNA (**1c**) is omitted for clarity because it overlaps the curve measured with SQ68. The optical density curve is the time integral of a thrombin generation curve. The final slope of this curve is the result of the amidolytic activity of the $\alpha_2$macroglobulin-thrombin complex.

optical density was monitored at 405 nm, see Fig. 1. From the optical density curve, the first derivative can be calculated giving the enzyme concentration during the measurement.

RESULTS AND CONCLUSION

The thrombin generation curve is a rich source of information on the coagulation process in an individual plasma sample. With the usual subsampling techniques (22), the experimental effort to obtain such a curve prohibits its application to plasma samples of individual patients in the clinical laboratory. The continuous method published earlier (1), allows the registration of a thrombin generation curve in a single sample by optical monitoring of the thrombin-dependent splitting of a chromogenic substrate. This method lends itself to adaptation to laboratory automatons. It

requires thrombin substrates that are not exhausted by the thrombin generation in a plasma sample. Current substrates such as S2238 (Kabi) do not fulfill this requirement but another substrate, SQ68 (Serbio) did. In search for specific thrombin substrates that are specific but nevertheless are converted slowly, a series of 25 peptide *p*-nitroanilides with H-D-Phe-Pip-Arg-pNA and CH_3O-CO-CH_2-CO-Aib-Arg-pNA as lead structures was synthesized. Classical solution methods were used and good yields were obtained.

Substrates containing the H-D-Phe-Pip(Pro) sequence are hydrolyzed by thrombin even when neutral or acidic amino acid side chains are present at position P1. However, the efficient binding interaction of H-D-Phe-Pip(Pro) with thrombin results in K_m values that were too low for our purpose. Varying P2/P3 residues appear to be the method of choice to arrive at substrates with higher K_m values. In this study the amino acid at P1 was always an arginine residue, which ensures optimal interaction with the S1 specificity pocket. The amino acid residue at P2 was systematically changed, incorporating: glycine, alanine and valine. This resulted in a gradual increasing occupancy of the hydrophobic binding pocket, leading to more important interaction and thus higher suitability for our purposes. The P3 modification was either omitted (a free N-terminus) or modified by a malonic acid derivative in which the methylene protons were changed for methyl- or ethyl groups. This resulted in fairly apolar aliphatic side chains which do not interact optimally with the aryl-binding site of thrombin. To achieve better interaction with the aryl-binding site, the N-terminus was modified by the introduction of an aromatic function. For this purpose the *p*-toluenesulfonyl- and β-naphthalenesulfonyl function were chosen (see table I). This however, did not result in compounds that could be hydrolyzed by thrombin. The similarity of compounds **3e** and **3f** to NAPAP suggests a similar binding mode in which the anilide bond points away from the Ser195 side chain.

In the continuous assay we need substrates which do not interact with factor Xa to minimize inhibition of thrombin formation and unwanted substrate hydrolysis by factor Xa. As can be seen from table II, compounds **1b**, **1c**, **3d** and **6** appear to be suitable to be used as substrates in the continuous thrombin assay. A detailed biochemical evaluation of the applicability of these substrates in the continuous thrombin assay will be published elsewhere.

Abbreviations

Aib: amino*iso*butyric acid (α-methylalanine); DCC: N,N'-dicyclohexylcarbodiimide; DCU: N,N'-dicyclohexylurea; DEMZ: diethylmalonic acid monomethyl ester; DIPEA: N,N-di*iso*propylethylamine; DMF: N,N-dimethylformamide; DMMZ: dimethylmalonic acid monomethyl ester; EtOAc: ethylacetate; MMZ: methylmalonic acid monomethyl ester; MZ: malonic acid monomethyl ester; pNA: *para*-nitroaniline; Nas: 2-naphthalenesulfonyl; Nle: norleucine; NMM: N-methylmorpholine; Orn: ornithine; Pip: pipecolic acid (homoproline); Tris: tris(hydroxymethyl)aminomethane; Tos: 4-toluenesulfonyl; UV: ultra-violet.

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